2014

Cul3 Ubiquitin Ligase and Ctb73 Protein Interactions

Lacey R. Royer
Portland State University

Cul3 Ubiquitin Ligase and Ctb73 Protein Interactions

By

Lacey Royer

An Undergraduate Thesis Submitted in Partial Fulfillment of the Requirements for the degree of

Bachelor of Science

in

University Honors

and

Micro/Molecular Biology

Thesis Adviser

Jeffrey Singer, Ph.D.

Portland State University

2014

Table of Contents Page

Acknowledgements

I would like to thank, first and foremost, Dr. Jeff Singer for taking the time to be my thesis advisor and for allowing me to take part in the use of his laboratory and equipment. The experience I have had learning from him and his graduate students was, by far, one of the most rewarding experiences I have had during my college career. A huge and sincere thank you goes to graduate students Brittney Davidge and Jennifer Mitchell; they were so helpful and welcoming. I could not have asked for a better lab group to work with.

I would also like to thank the Honors Department at Portland State University for allowing me to be a part of the excellence that is known to be the university honors program. If it weren't for the honors program, the opportunity to perform independent research might not have occurred; for that I am grateful. I have met so many fantastic people and have learned so much through my studies and laboratory research. I feel that these experiences will only enhance my future studies and endeavors.

Introduction

Ubiquitination

Proper regulation of proteins in the cell is important for cellular processes such as cell cycle and division, DNA transcription and repair, and differentiation and development. Ubiquitination is a fundamental biochemical process, which controls numerous aspects of protein function, such as degradation, protein-protein interaction and subcellular localization (Sadowski et. al, 2010). Ubiquitin (Ub) is a highly conserved 76-amino-acid-polypeptide small regulatory protein containing 7 lysines found only in eukaryotic cells (Figure 1A). Ubiquitin attachment to certain lysine residues generates diverse substrate-ubiquitin structures, targeting proteins to different cellular compartments and affecting cell physiology (Hicke, 1999). Poly-ubiquitin chains are built by formation of an isopeptide bond between Glycine 76 of one ubiquitin to the epsilon amide group of one of the seven potential lysines (K6, K11, K27, K29, K33, K48 or K63) of the preceding ubiquitin (Figure 1B). The ability to generate diverse substrate-ubiquitin structures is important for targeting proteins to different fates. For example, monoubiquitination can regulate DNA repair and gene expression. Polyubiquitination through Ub K48 generally targets proteins for

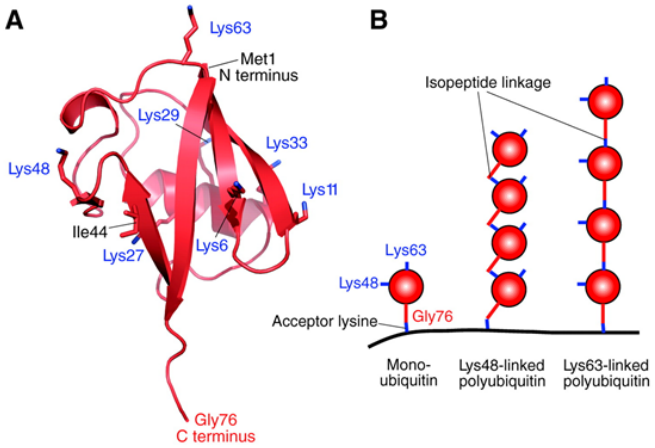


Figure 1. *Ubiquitin, isopeptide linkage and chain conformation variations (Traub et. al, 2007).*

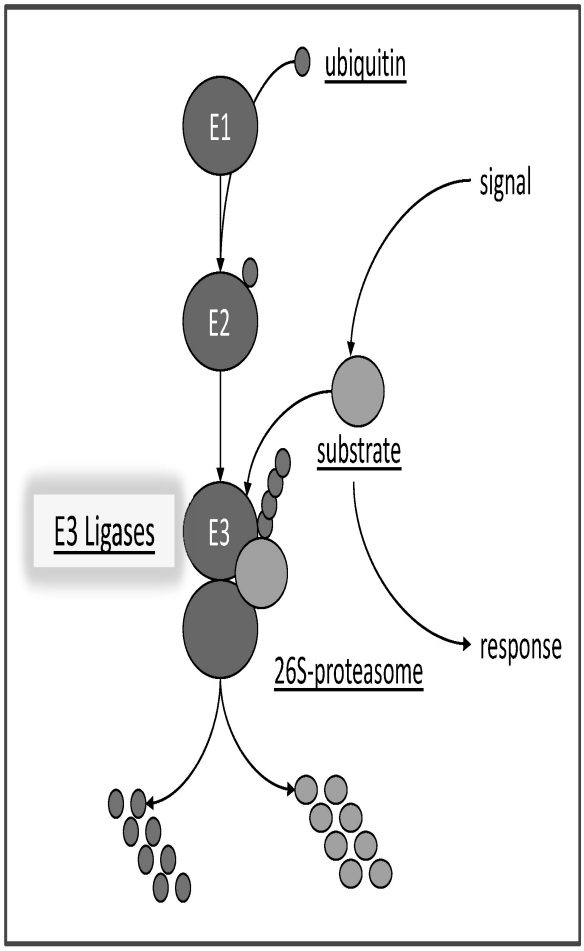
A. Seven lysines available for conjugation of Ubiquitin.

B. Isopeptide bond formation of K48 and K68.

proteasomal degradation, while K63-linked Ub chains can regulate kinase activation, DNA damage tolerance, signal transduction and endocytosis (Sadowski, et. al, 2010).

The ubiquitination process occurs in three steps (Figure 2): ubiquitin activation via an E1 activating enzyme, transfer to E2 conjugating enzyme, and covalent attachment via E2 and the target substrate. It has been shown that E3 ligases function as scaffold proteins, specifically binding substrate proteins and specific E2s; that ubiquitin is then transferred directly from E2s to substrates (Scheffner et. al, 1999). It has also been shown that the chemical properties of the active site of each E3 ligase function in a very specific way, targeting specific lysine residues and catalyzing a specific ubiquitination pathway and specific substrates. It is the specificity in the E2/E3 combinations that result in the formation of linkage specific ubiquitin chains.

Figure 2. *Ubiquitin Pathway* (Hellman et. al, 2002).



E3 Ligase Families

There are two main classes of E3 ligases that differ based on their E2 binding domains and mechanism of action: RING (Really Interesting New Gene) E3 ligases act as scaffolds for E2 enzymes, facilitating the direct transfer of ubiquitin on to the substrate; HECT (Homologous to

E6-AP C-terminus) E3 ligases covalently attach to ubiquitin before it is transferred to the substrate. The RING E3 ligases are further sub-divided into the monomeric or simple RING type and the multimeric type, which includes the Cullin family (Wilkie et. al, 2012). Most Eukaryotic genomes encode at least six cullins (Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5) and are crucial for a variety of biological processes (Chen et al., 2009). Cullins share a homologous C terminus that binds the RING-finger protein Rbx1, but are heterogenous when it comes to the N-terminal substrate recruiting domain. The best understood of the Cullin superfamily is Cul 1 and its involvement in the Cul1-Skp1-F-box protein complex. Skp1 serves as an adaptor that simultaneously binds sequences near the N-terminus Cul1 and the F-box motif of an F-box protein which, in turn, recruits substrates through additional protein-interaction domains and facilitating ubiquitination via the C-terminus of Cul1 (Figure 3). Other Cullins such

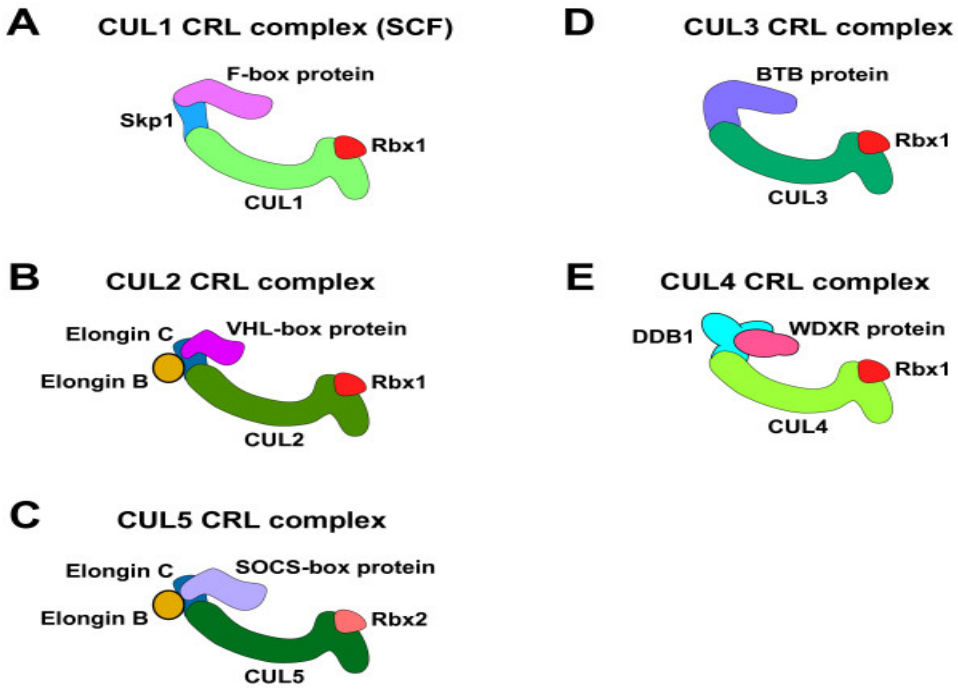


Figure 3. *A schematic representation of cullin family ubiquitin ligases and substrate adaptors. Cul1 binds F-box proteins (A) while Cul3 binds BTB proteins (D).* (Bosu et al., 2008).

as Cul2 and Cul5 protein complexes are known to share a similar organization, varying in the protein specificity at the N-terminus (Zhuang et al., 2009).

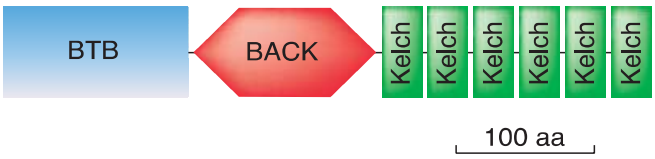
Cul3 and BTB Proteins

Cul3 is just beginning to be understood and has been found to be involved in a variety of ctitical cellular processes such as regulation of the cell cycle. The Cul3 ubiquitin ligase is known to target proteins for poly-ubiquitination that play a critical role in the cell cycle (Cummings et al., 2008). A protein domain that has been highly conserved and found in many proteins that function as substrate adaptors for Cul3 is the BTB (Brick-Brack, Tram-Trak, and Broad Complex) domain (Romero et al., 2013). Like all Cullins, Cul3 binds to an Rbx1 protein on its C terminus but unlike other Cullins, Cul 3 binds the BTB domain of the BTB containing protein on its N terminus (Figure 3). BTB is a protein interaction/dimerization domain that is structurally homologous to the cullin- binding region of Skp1 and that binds Cul3 via motifs analogous to those in the Skp1-Cul1 complex (Zhuang et al., 2009). Altogether, this forms the BTB-Cul3-Rbx1 (BCR) complex. There have been more than 200 BTB proteins characterized in humans (Singer et, al.,) and these proteins harbor additional protein-protein interaction modules signifying the importance of BTB domain containing proteins (Chen et al., 2009). BTB protein family can be subdivided into BTB-zinc finger (BTB-ZF), BTB-BACK-kelch (BBK), MATH-BTB, BTB-NPH3, Kelch family (KLHL), and Kelch repeat and BTB domain- containing proteins (KBTBD) subfamilies of proteins, among others; it is through the multiplicity in protein interacting domains that contributes to their ability to act as substrate adaptors for Cul3 mediated ubiquitination (Anderica-Romero et al., 2013).

Ctb73

A novel BTB domain containing protein that has been shown to interact with Cul3 is Ctb73, or BTBD. Ctb73 has a BACK and Kelch domain (Figure 4), belonging it to the BBK class of BTB domain containing proteins . The BACK (**B**TB **a**nd **C**-terminal **K**elch) domain was identified as a conserved sequence motif in proteins containing both BTB and Kelch motifs; based on its predicted secondary structure and proximity to the BTB domain, the BACK domain is hypothesized to be involved in substrate orientation for ubiquitin conjugation within BTB-Cul3 ubiquitin ligase complexes. It is possible that the BACK domain functions to position the kelch-motif beta propeller and its bound substrate in the Cul3 E3 complexes. BBK proteins are found predominantly in vertebrates and there are 53 found in humans (Stogios et. al., 2004).

Figure 4. *Architecture of BBK proteins* (Stogios et al., 2004).



Some members of the BTB-Kelch domain subfamily are known to interact directly with actin or to regulate the actin cytoskeleton, thereby affecting cell-cell interactions, cell-substrate interactions, and cell migration (Gray et al., 2009). Since Cul3 is the most recently identified and least understood of the Cullin family ubiquitin ligases and are known to interact with an array of BTB domain containing proteins, it is important to study the interactions between Cul3 and BTB proteins; specifically how those proteins function as adaptors for substrate ubiquitination. BTB proteins work as Cul3 substrate adaptors; since there are approximately 200 BTB proteins

encoded in the human genome, this implies the vast array of possible substrates for Cul3 ubiquitination (Cummings et al., 2008). This ubiquitination process is highly orchestrated; every protein involved in the complex is critical for the regulation of specific substrates. In order to understand the importance of Cul3, we must be able to understand all of their protein interactions.

A novel BTB domain containing protein has been found that contains a BACK and kelch domain, Ctb73 or BTBD. It is not known whether or not Ctb73 works as a substrate adaptor for Cul3 or how it binds to Cul3. The purpose of this experiment is to determine if Ctb73 binds to Cul3 through any of the protein-interacting domains. In order to do this, HEK 293 cells will be co-transfected with 3X FLAG-tagged Cul3 and one of the following Myc- tagged Ctb protein plasmids: Ctb73 wildtype (WT), Ctb73 **Δ**BTB with its BTB domain mutated, Ctb73 **Δ**BACK with its BACK domain mutated, and Ctb73 **Δ**BTB-BACK with its BTB and BACK domains mutated. The cells expressing these proteins will be harvested and lysed 48 hours later. Expression levels will be determined using immunoblotting for Myc and FLAG antibodies. A FLAG antibody immunoprecipitation assay and a Myc antibody Western blot will be performed in order to detect binding of the Ctb 73 proteins and Cul3. Since it has been shown that the BTB domain is the domain in a BTB protein that interacts with Cul3, both of the Ctb73 proteins with a mutated BTB domain are not expected to bind to Cul3. However, it is expected that Ctb73 wildtype and Ctb73 with mutated BACK domain only will bind to Cul3. This will be shown by the absence of banding patterns in the lanes correlating to Cul3 plus Ctb 73ΔBTB and Cu3 plus Ctb73ΔBTB-BACK in the FLAG immunoprecipitation assay and Myc Western blot.

Methods

Plasmid Preparation and Purification

3X-FLAG tagged Cul3 transformed ampicillin resistant *E. coli* were grown in lysogeny broth (LB) media overnight. Bacterial cells were harvested and lysed, followed by plasmid DNA purification.

Transfection of HEK 293 Cells

Cells from the human embryonic kidney 293 cell line (HEK 293) were allowed to grow to confluency on p90 plates then spilt for transfection on p60 plates. The cells were co-transfected by calcium phosphate precipitation with purified plasmids containing a 3X-FLAG tagged Cul3 and either one of the Myc-tagged Ctb73 plasmids: Ctb73 WT, Ctb73ΔBTB, Ctb73ΔBACK, or Ctb73ΔBTB-BACK, incubated for 48 hours before harvest and cell lysis.

Immunoprecipitation and Immunoblotting

An immunoprecipitation assay was used to precipitate or "pull down" FLAG tagged Cul3 and whatever protein is bound to Cul3 for each cell extract. To see if there was a Ctb73 protein bound to Cul3, Western blotting by immobilizing the proteins on a membrane and probing them by complexing them with primary antibodies followed by horseradish peroxidase (HRP) conjugated secondary antibodies. The Western blot was developed using an enhanced chemiluminescence reaction (ECL).

Results

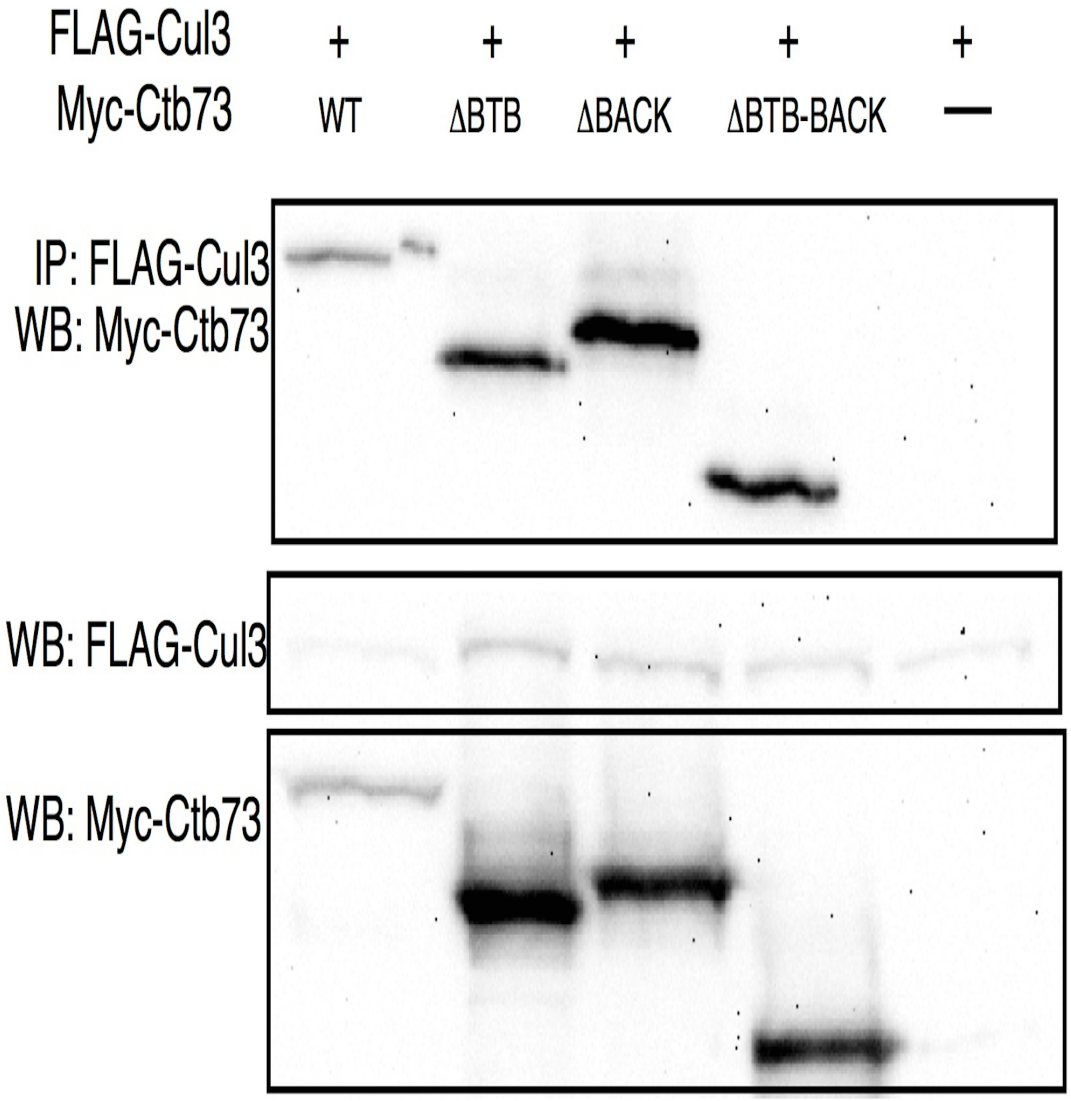


Figure 5. FLAG Immunoprecipitation assay and Myc Western blotting was performed to detect protein binding. Expression levels of Cul3 and Ctb73 plasmids were detected using western blot analysis using mouse Flag and mouse Myc antibodies, respectively. An immunoprecipitation assay using mouse FLAG antibodies precipitated out Cul3 proteins then binding of Ctb73 proteins to Cul3 was analyzed via probing of Western blot using rabbit Myc antibodies. It was shown that all Ctb 73 proteins bound to Cul3. Expression levels of Cul3 were much lower, as shown in figure 5, than that of all the Ctb73 plasmids; due to this, the results from the mouse FLAG immunoprecipitation assay and rabbit Myc Western blot are unreliable.

Discussion

Ubiquitination is a fundamental biochemical process, which controls numerous aspects of protein function, such as degradation, protein-protein interaction and subcellular localization (Sadowski et. al, 2010). This occurs through a series of steps and involves an ubiquitin-activating enzyme, E1 forms a thioester bond with ubiquitin, a highly conserved 76-amino acid protein. This reaction allows subsequent binding of ubiquitin to a ubiquitin conjugating enzyme, E2, followed by the formation of an isopeptide bond between the carboxy-terminus of ubiquitin and a lysine residue on the substrate protein by a ubiquitin ligase, E3.

The focus of this experiment was to determine protein interactions between an E3 ubiquitin ligase, Cul3 and a BTB protein, Ctb73. BTB proteins have been found to work as substrate adaptors of Cul3, interacting with the substrate and Cul3; facilitating the ubiquitination process. There have been more than 200 BTB proteins found, implying the significance of these proteins in normal cell functioning. Ctb73 is a novel BTB protein that contains a BACK and Kelch domain. BTB domain containing proteins are known to bind to Cul3 via the BTB domain; whether binding occurs through other domains is not yet clear. In order to determine whether or not Ctb73 binds to Cul3 through the BACK domain, located in the middle of the BTB and Kelch domains, HEK 293 cells were transfected on p60 plates with 3X-FLAG Cul3 and one of the Myc-tagged Ctb 73 plasmids: WT, ΔBTB, ΔBACK, and ΔBTB-BACK. Expression levels of Cul3 and Ctb73 proteins were determined by probing Western blots with mouse FLAG antibody and mouse Myc antibody, respectively. Mouse FLAG antibody immunoprecipitation assay was used to bring down Cul3 of cell lysates and a Western blot was probed using rabbit

Myc antibody to detect Ctb73 binding to Cul3 (Figure 5). It was expected that, since BTB domain of BTB domain containing proteins has been shown to bind Cul3, there would not be binding between Cul3 and both the Ctb73 BTB domain mutants, Ctb73ΔBTB and Ctb73ΔBTB-BACK. However, it was expected that the Ctb73 WT and Ctb73ΔBACK proteins would bind to Cul3. The results of this experiment show that all Ctb73 proteins bind to Cul3. It is believed that since Cul3 expression levels were much lower than that of Ctb73 expression levels, this result is due to high levels of background and therefore, not conclusive data.

For future experiments, adjusting levels of protein expression could obtain more reliable results by either changing the amount of DNA transfected or the amount of cell lysate used for immunoprecipitation assay. Even levels of expression are important for accurate binding detection. Once protein interactions of Cul3 and Ctb73 have been determined, the next step would be to determine binding specificity between Ctb73 and Cul3 specific substrates. HEK 293 cells could be transfected with differently tagged plasmids then immunoprecipitation assays and Western blots would be used to detect binding in the same way as the current experiment. Another step towards the understanding of Cul3 and Ctb73 protein interactions would be cellular localization studies to determine where in the cell Cul3 and Ctb73 interact. This would further our knowledge of possible implications and functions of Cul3 and Ctb73 and their importance for canonical cellular functioning.

Literature Cited

Anderica-Romero, Ana C., Irma G. Gonzalez-Herrera, and Abel Santamaria. "Cullin 3 as a Novel Target in Diverse Pathologies." *Redox Biology*. 2013.

Bosu, Dimple R., and Edward T. Kipreos. "Cullin-RING Ubiquitin Ligases: Global Regulation and Activation Cycles." *Cell Division* 3.1 (2008): 7.

Chen, Yuezhou, Zhenxiao Yang, Min Meng, Yue Zhao, Na Dong, Hongming Yan, Liping Liu, Mingxiao Ding, H. Benjamin Peng, and Feng Shao. "Cullin Mediates Degradation of RhoA through Evolutionarily Conserved BTB Adaptors to Control Actin Cytoskeleton Structure and Cell Movement." *Molecular Cell* 35.6 (2009): 841-55.

Cummings, C. M., C. A. Bentley, S. A. Perdue, P. W. Baas, and J. D. Singer. "The Cul3/Klhdc5 E3 Ligase Regulates P60/Katanin and Is Required for Normal Mitosis in Mammalian Cells." *Journal of Biological Chemistry* 284.17 (2008): 11663-1675.

Gray, C. H., L. C. Mcgarry, H. J. Spence, A. Riboldi-Tunnicliffe, and B. W. Ozanne. "Novel -Propeller of the BTB-Kelch Protein Krp1 Provides a Binding Site for Lasp-1 That Is Necessary for Pseudopodial Extension." *Journal of Biological Chemistry* 284.44 (2009): 30498-0507.

Hellman, Hanjo and Estelle, Mark. “Plant Development: Regulation by Protein Degradation.” *Science.* 297.5582 (2002): 793-797.

Hicke, Linda. "Gettin’ down with Ubiquitin: Turning off Cell-surface Receptors, Transporters and Channels." *Trends in Cell Biology* 9.3 (1999): 107-12. *Science Direct*.

Sadowski, Martin, and Boris Sarcevic. "Mechanisms of Mono- and Poly-ubiquitination: Ubiquitination Specificity Depends on Compatibility between the E2 Catalytic Core and Amino Acid Residues Proximal to the Lysine." *Cell Division* 5.1 (2010): 19.

Scheffner, M., U. Nuber, and J.M. Huibregtse. "Protein Ubiquitination Involving E1-E2-E3 Enzyme Ubiquitin Thioester Cascade." *Nature*: *National Center for Biotechnology Information*. U.S. National Library of Medicine. 1995.

Traub, Linton M. and Lukacs, Gergley L. "Decoding Ubiquitin Sorting Signals for Clathrin-Dependent Endocytosis by CLASPs." *Journal of Cell Science*. 2007.

Wilkie, Neil and Davies, Steve. "Ubiquitination, E3 ligases and Drug Discovery Novel Technologies for a Challenging Pathway." *Drug Discovery World.* 2012.

Zhuang, Min, Matthew F. Calabrese, Jiang Liu, M. Brett Waddell, Amanda Nourse, Michal Hammel, Darcie J. Miller, Helen Walden, David M. Duda, Steven N. Seyedin, Timothy Hoggard, J. Wade Harper, Kevin P. White, and Brenda A. Schulman. "Structures of SPOP-Substrate Complexes: Insights into Molecular Architectures of BTB-Cul3 Ubiquitin Ligases." *Molecular Cell* 36.1 (2009): 39-50.